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A spectrophotometric assay for feruloyl esterases

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Abstract

We have developed a spectrophotometric assay for the quantitative determination of feruloyl esterase activity based on release of 4-nitrophenol from a novel substrate, 4-nitrophenyl ferulate in an emulsion of Triton X-100 in aqueous buffer solution. The release of 4-nitrophenol was linear with reaction time at an early stage of the reaction with various esterase preparations. The method proposed here is accurate, rapid, and easy to perform. © 2002 Elsevier Science (USA). All rights reserved.

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Cinnamoyl (phenolic acid) esters represent an integral part of plant materials. Their probable role is to decrease cell wall biodegradability and regulate the growth by cross-linking of cell wall polymers. The content of cinnamoyl esters in plant material can reach up to 2.5% (w/w) of cell walls. Therefore, it is not surprising that enzymes involved in their degradation are of growing interest to many researchers. Cinnamic acids are released from plant polysaccharides by cinnamoyl esterases, mainly feruloyl and *p*-coumaroyl esterases. Potential applications of these enzymes include complete degradation of plant material [1], isolation of phenolic acids as fine chemicals from agro-industrial wastes [2], and evaluation and improvement of sensory properties of wine [3].

The most frequently studied cinnamoyl esterase is feruloyl esterase (FE).[1] Several methods have been reported for measuring its activity. Most of them are based on HPLC techniques, using enzymatic hydrolysis of hydroxycinnamic esters [3,4], plant polysaccharides [1,5,6], their fragments [7] and fragment analogues [8], as well as chlorogenic acid or hydroxycinnamic tartrate-containing materials [3]. Unfortunately, these HPLC methods require expensive equipment, are time-consuming, and are not suitable for rapid analysis of large numbers of samples. Some of these methods require the isolation of natural substrates, which adds another sometimes laborious step. Capillary zone electrophoresis [9] and gas chromatography [10] have also been applied to FE assays using natural substrates, their analogues and hydroxycinnamic methyl esters, but these methods possess similar disadvantages.

Spectrophotometric analyses for cinnamoyl esterase activity described in the literature rely on the use of differences in spectral properties of free hydroxycinnamic acid and its natural esters [11,12] or their analogues [8]. Such methods measure relatively low changes of absorbance and have not become generally adopted.

In this study we describe a new spectrophotometric method for determining FE activity using 4-nitrophenyl ferulate (4NPF), a cheap substrate which is easy to prepare [13]. The method is simple and is based on the measurement of 4-nitrophenol (4NP) released upon enzyme action. The main drawback in using such a substrate is its solubility in aqueous buffer solutions. Inspired by the method for lipase assays [14], we used dimethyl sulfoxide and a detergent, Triton X-100, to

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[1] *Abbreviations used:* FE, feruloyl esterase; 4NP, 4-nitrophenol; 4NPF, 4-nitrophenyl ferulate; 4NPL, 4-nitrophenyl laurate.

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create transparent emulsions stable for several hours, enabling us to measure feruloyl esterase activity spectrophotometrically.

Materials and methods

Chemicals

4-Nitrophenyl ferulate was prepared according to the chemoenzymatic procedure described by Mastihubová et al. [13]. Ethyl ferulate was from Sigma Chemical (St. Louis, MO) and Triton X-100 was from Koch-Light Laboratories (Colnbrook, Buckinghamshire, England). All other chemicals were purchased from commercial sources and of analytical or HPLC grade. Precoated silica gel 60 plates, 0.25-mm layer thickness, with UV_{254} indicator were from Merck (Darmstadt, Germany).

Enzyme preparations

Enzyme preparation Cytolase M102 was a generous gift from DSM Food Specialties (Wilmington, DE). Lipase A, Lipase PS30, Lipase M, Proleather, and Protease N were gifts from Amano Enzyme, USA (Lombard, IL). Porcine pancreatic lipase and chymotrypsin were from Sigma. *Trichoderma reesei* RUT C30 was cultivated on 1% cellulose medium according to Biely et al. [15]. The fungus was grown in 2800-ml Fernbach flasks containing 700 ml of medium for 4–5 days at 28 °C on a rotary shaker at 200 rpm.

Cytolase M102 and *T. reesei* RUT C30 culture filtrate were desalted by ultrafiltration using a Millipore Pellicon system (Millipore, Bedford, MA) with a 10^4 molecular weight cut-off membrane, and subsequently freeze-dried. Prior to use, lyophilized enzyme preparations were dissolved in 0.05 M sodium acetate buffer, pH 5.4.

All lipases and both proteases from Amano were dialyzed overnight against 0.05 M potassium phosphate buffer, pH 6.5, using membrane tubing (Spectrapor, Houston, TX) with a molecular weight cut-off of 3500.

All enzyme preparations were centrifuged for 5 min at 13,800*g* before use. The amounts of enzymes in reaction mixtures are expressed as protein content by weight.

Apparatus

High-performance liquid chromatography consisted of a Spectra-Physics SP 8800 pump system equipped with a solvent mixer, a Rheodyne injector with a 20-μl loop, and a Microsorb MV C-18 reverse-phase column. Effluent was monitored by UV absorbance on a Rainin Dynamax spectrophotometric detector (Rainin Instrument, Woburn, MA) at 254 nm.

Enzyme activities

Feruloyl esterase—Spectrophotometric assay (standard method). Enzyme activity was determined by measuring the production of 4NP from 4NPF. The substrate solution was prepared by mixing 9 vol of 0.1 M potassium phosphate buffer solution, pH 6.5, containing 2.5% Triton X-100 with 1 vol of 10.5 mM 4NPF in DMSO followed by immediate vortexing. This buffer–4NPF solution was prepared freshly before analysis; the DMSO solution of 4NPF was prepared within 24 h and kept at room temperature. Lower concentrations of Triton X-100 were effective at solubilizing the substrate, but solutions were not stable. For periods up to 1 day, the minimum effective Triton X-100 concentration should be 2.5%. Reagent blanks are strongly recommended, as the emulsions occasionally exhibit variations in absorbance. The reaction mixture comprised 0.1 ml enzyme and 2 ml substrate solutions. The change of absorbance was read at 410 nm in 10-mm pathlength polystyrene cuvettes. In a control sample, the enzyme was replaced by appropriate buffer. One unit of enzyme activity is defined as the amount of enzyme releasing 1 μmol of 4NP from 4NPF in 1 min. The apparent extinction coefficient used in calculations represents the difference between extinction coefficients of 4NP and 4NPF (see Results).

Feruloyl esterase—HPLC assay. The assay was based on the measurement of ferulic acid released from ethyl ferulate. One volume of enzyme solution was mixed with 3 vol of 1.33 mM ethyl ferulate in 0.05 M potassium phosphate buffer, pH 6.5. Both solutions were preheated to 40 °C before mixing. The final mixture was incubated at the same temperature. At various time intervals, 0.3-ml aliquots of the reaction mixture were withdrawn and mixed with 0.1 ml of 0.35 M H_2SO_4 to stop the reaction. This was followed by the addition of 0.3 ml of 1.0 mM benzoic acid as internal standard and 0.1 ml of 0.7 M NaOH. The solution was mixed by vortexing, passed through a 0.45-μm syringe filter, and analyzed by HPLC. Using the apparatus previously described, acidic components of the samples were eluted with a mixture of water:acetic acid:1-butanol (350:1:7, vol/vol/vol) with a flow rate of 1 ml/min. A linear gradient of methanol (ramped up to 100% methanol in 5 min) was used to wash off the unreacted ethyl ferulate. All ethyl ferulate, ferulic acid, and benzoic acid were dissolved in minimal volume of ethanol prior to mixing with buffer or water.

Feruloyl esterase—Thin-layer chromatographic estimation. The reaction mixture was prepared and incubated as in the HPLC assay. After 8 h, 3 vol of reaction mixtures was mixed with 1 vol of 0.35 M H_2SO_4. At least 7 μl of such mixtures was applied to precoated silica gel plates with UV_{254} indicator and developed with a solvent mixture of ethyl acetate:benzene:2-propanol (2:1:0.1, vol/vol/vol). Spots for ferulic acid ($R_f = 0.19$) and ethyl ferulate ($R_f = 0.80$) were detected by UV absorbance.

Other enzyme assays. Lipase activities were estimated by a modification of the method described by Kordel et al. [14] using 4-nitrophenyl laurate (4NPL) as substrate: 0.05 ml of enzyme was mixed with 1.0 ml of 1.5 mM solution of 4NPL. The substrate was freshly prepared by diluting a 15 mM 4NPL solution in DMSO with 9 vol 0.05 M Tris–HCl buffer, pH 8.0, containing 0.4% Triton X-100 and 0.1% (w/v) gum arabic. The reaction mixture was preincubated at 37 °C and the increase in absorbance at 410 nm was measured against a blank containing buffer instead of the enzyme. Enzyme activity was calculated from slopes of absorbance vs time. One unit of lipase activity is defined as the amount of enzyme that releases 1 μmol of 4NP in 1 min.

Acetyl esterase activities were measured on 4NP acetate according to Biely et al. [16]. Proteolytic activities were determined spectrophotometrically with azocasein as a substrate according to Cotta and Hespell [17]. The reaction was stopped at various time intervals and the proteolytic activity was expressed as change of absorbance at 440 nm in 1 min.

Other methods

Protein contents were determined according to the method published by Bradford [18].

Results

Standard curve for 4NP and 4NPF and calculation of product concentration

Solutions of 4NP or 4NPF under reaction conditions resulted in linear standard curves exhibiting correlation factors of 0.9997 and 0.9985, respectively. The absorbance of ferulic acid was minimal (0.02 for 2 mM solution) and was neglected in the activity calculations. Extinction coefficients for 4NP and 4NPF in reaction conditions were 2.034×10^3 and $0.300 \times 10^3\,\mathrm{dm^3\,mol^{-1}\,cm^{-1}}$, respectively. This was used to give corrected values for concentration according to the equation $A_{410} = \varepsilon_p l[P] + \varepsilon_s l[S] = \varepsilon_p l[P] + \varepsilon_s l([S]_0 - [P]) = \varepsilon_p l[P] - \varepsilon_s l[P] + \varepsilon_p l[S]_0$, where l is the path length, ε_p is the extinction coefficient of the product 4NP, ε_s is the extinction coefficient of the substrate 4NPF, and [P], [S], and $[S]_0$ represent the product concentration, substrate concentration, and initial substrate concentration, respectively. If absorbance is measured against a substrate-containing blank, the expression simplifies to $A_{410} = (\varepsilon_p - \varepsilon_s)[P]l$, where $(\varepsilon_p - \varepsilon_s) = 1.734 \times 10^3\,\mathrm{dm^3\ mol^{-1}\,cm^{-1}}$.

Optimization of reaction conditions

The reaction conditions were optimized using the commercial enzyme preparation Cytolase M102, a complex of pectinase and cellulase produced by *Aspergillus niger* which also contains feruloyl esterase activity.

Using Cytolase M102, we examined the effect of 4NPF concentration on the rate of 4-nitrophenol released. As Fig. 1 indicates, saturation occurred at a substrate concentration exceeding approximately 2.0 mM. Experience shows us that the emulsion system is not stable over longer periods at 4NPF concentrations above 1.3 mM, so an initial substrate concentration of 1.00 mM in reaction mixtures was chosen for further experiments. For practical reasons the reaction temperature was set up to ambient temperature (25 °C), as when nonthermostated photometer cells are to be used.

Kinetics of the assay at chosen enzyme concentrations had shown that the reaction is linear up to absorbance $A_{410} = 0.75$, or within 7 h of incubation (Figs. 2A and B).

Following the rate of 4NP release from 4NPF (Fig. 3) it can be seen that the rates measured by this assay correspond to enzyme concentration in a linear fashion up to a Cytolase M102 concentration of 2.821 mg, corresponding to an activity of 0.215 U in the reaction mixture. To test the feasibility of our assay method we selected a range of commercial enzymes possessing various combinations of feruloyl esterase, acetyl esterase, lipase, and protease activities (Table 1). Feruloyl esterase was determined spectrophotometrically on 4NPF and by HPLC using ethyl ferulate as described above.

As seen in Table 1, the levels of feruloyl esterase activities among lipase preparations varied. Lipase PS30 exhibited no hydrolytic activity against 4NPF and low activity against ethyl ferulate. Low activities toward both substrates were also observed for Lipase PPL and Lipase M, but contrary to those results, the specific

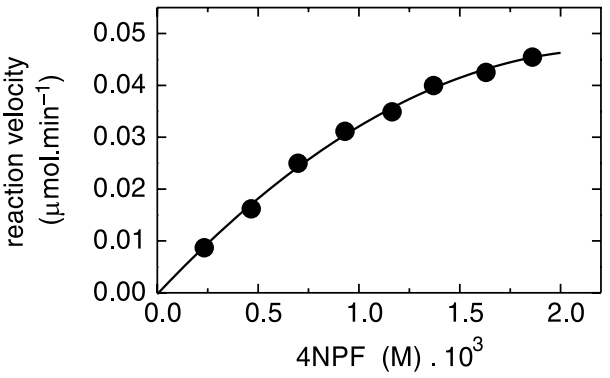

Fig. 1. Effect of substrate (4NPF) concentration on velocity of its hydrolysis by Cytolase M102. Incubations were carried out in a final volume of 2.1 ml containing 0.451 mg of Cytolase M102, 4NPF (0.233–1.860 mM), and 0.09 M potassium phosphate buffer, pH 6.5, containing 2.14% Triton X-100 and 9.52% DMSO at room temperature (~25 °C).

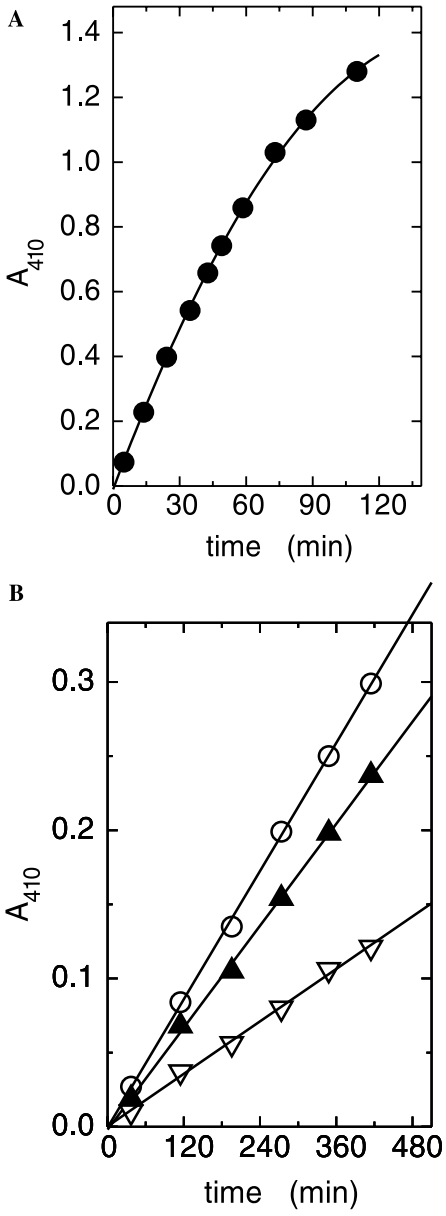

Fig. 2. Time course for the hydrolysis of 4NPF. Incubations were carried out in a final volume of 2.1 ml containing 1.0 mM 4NPF, 0.09 M potassium phosphate buffer, pH 6.5, containing 2.14% Triton X-100, 9.52% DMSO at room temperature (~25 °C), and Cytolase M102 in the following amounts: (**A**) (●) 265.0 μg; and, (**B**) (○) 13.3 μg; (▲) 10.6 μg, and (▽) 5.3 μg.

activities of Lipase A against both 4NPF and ethyl ferulate were the highest of all the enzymes studied.

Although all proteases possessed very low ethyl ferulate-hydrolyzing activities, two of them, Chymotrypsin and Proleather, hydrolyzed 4NPF at higher rates. The Sigma lipase PPL preparation exhibited high protease activity, comparable with those of preparations which were designated as proteases.

Cytolase M102 showed high feruloyl esterase activities on both 4NPF and ethyl ferulate, while the

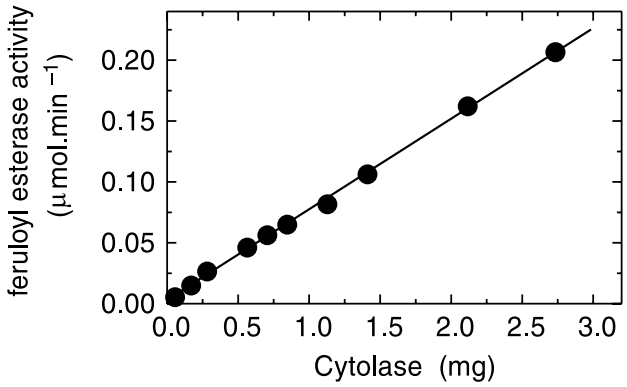

Fig. 3. Effect of enzyme concentration. Assays were carried out in a final volume of 2.1 ml containing various concentrations of Cytolase M102 (0.056–2.840 mg/assay), 1.0 mM 4NPF, and 0.09 M phosphate buffer, pH 6.5, 2.14% Triton X-100, and 9.52% DMSO at room temperature (~25 °C).

T. reesei enzyme preparation showed no activity against 4NPF and very low hydrolytic activity on ethyl ferulate.

The highest specific acetyl esterase activity was found in Lipase PS30, and high activities were also measured also in Lipases M and A and in *T. reesei* culture fluid.

Discussion

We have developed a new assay method for feruloyl esterase. In the assay, the amount of 4-nitrophenol liberated from a buffered emulsion of 4-nitrophenyl ferulate is measured spectrophotometrically. The method was optimized on a crude enzyme preparation containing feruloyl esterase activity and its feasibility was tested on nine enzyme preparations comprising various combinations of enzyme activities, including acetyl esterase, lipase, and protease.

Although higher activities were found by this new assay compared to the HPLC method using ethyl ferulate as a substrate, we cannot be certain whether this method is in error on the high side or whether the ethyl ferulate HPLC method errs on the low side. The advantage of this method compared to other assays lies in its simplicity. It is a simple spectrophotometric procedure and, unlike HPLC or GLC, requires no expensive laboratory equipment. It is also much more suitable when large numbers of samples must be analyzed. We find this technique to be especially useful in performing kinetic measurements.

Certain other considerations must be taken into account when the assay is used. Of greatest concern is its relative sensitivity to the presence of proteases. According to the results summarized in Table 1, proteases display significantly lower (often negligible) activity against ethyl ferulate than 4NPF. This

Table 1
Comparison of acetyl esterase (AE), lipase, protease, and feruloyl esterase (FAE) activities of various enzyme preparations used in the study

Enzyme source	Protein ($mg \cdot ml^{-1}$)	AE ($U \cdot mg^{-1}$)	Lipase ($mU \cdot mg^{-1}$)	Protease ($A_{440} \cdot min^{-1} \cdot mg^{-1}$)	FAE	
					EtFer ($mU \cdot mg^{-1}$)	4NPF ($mU \cdot mg^{-1}$)
Lipase A (Amano)	3.71 ± 0.053	2.6 ± 0.20	4.6 ± 0.3	0.021 ± 0.001	411 ± 25	1110 ± 54.2
Lipase PS30 (Amano)	0.148 ± 0.008	580 ± 44	$1.35 \times 10^6 \pm 0.09 \times 10^6$	—	0.28 ± 0.03	—
Lipase M (Amano)	2.000 ± 0.001	4.0 ± 0.32	103 ± 6.6	0.003 ± 0.001	0.11 ± 0.01	5.4 ± 0.4
Lipase PPL (Sigma)	2.7 ± 0.12	0.20 ± 0.01	9.9 ± 0.3	0.90 ± 0.048	0.03 ± 0.01	5.0 ± 0.3
Proleather (Amano)	1.88 ± 0.024	0.64 ± 0.06	25.7 ± 0.2	6.6 ± 0.71	0.06 ± 0.01	25 ± 2.2
Protease N (Amano)	10.2 ± 0.21	<0.02	0.6 ± 0.1	1.6 ± 0.10	<0.01	0.6 ± 0.1
Chymotrypsin (Sigma)	2.2 ± 0.20	—	13.8 ± 0.7	1.21 ± 0.092	0.24 ± 0.01	26 ± 1.7
Cytolase M102 (DSM Food Specialties)	14.3 ± 0.25	0.35 ± 0.02	3.4 ± 0.2	<0.001	77 ± 3.0	75 ± 3.2
T. reesei RUT C (dialyzed medium)	5.0 ± 0.43	1.54 ± 0.03	330 ± 29.3	<0.002	0.08 ± 0.01	—

Note. Values are given as averages of at least two analyses ± SD.

phenomenon is probably caused by the general esterase activity of proteases which is exhibited against many 4-nitrophenyl esters and should be taken into account when higher protease activities are present. Chymotrypsin and Proleather proteases exhibited similar rates for hydrolysis of ferulates, suggesting their higher specificity/selectivity for cinnamoyl derivatives. This may be explained by the high preference of subtilisin [19] and porcine chymotrypsin [20] for the hydrolysis of peptide bonds involving aromatic amino acids. The presence of protease activity may explain the high rate of hydrolysis of 4NPF and negligible rate of ethyl ferulate hydrolysis by porcine pancreatic lipase. For this reason, it is recommended that ethyl ferulate hydrolysis should be detected by the thin-layer chromatography method to confirm the presence of feruloyl esterase in unknown samples. It is also recommended that protease assays be performed to eliminate the possibility of interference from this type of activity in nonroutine samples. Attempts to minimize protease interference by providing an excess of "sacrificial" proteins such as bovine serum albumin were unsuccessful, as the relative effects on protease activity were unpredictable and varied greatly among preparations. Likewise, the use of protease inhibitors cannot be recommended, as their relative effect on various proteases and other esterases is too variable.

Using several enzyme preparations, we have demonstrated that this method can readily distinguish between acetyl esterase and feruloyl esterase activities. Among the samples analyzed, no correlation between levels of acetyl esterase and feruloyl esterase activity was found. This can be confirmed by comparing data obtained for certain lipases and for Cytolase M102 and *T. reesei* culture fluid. Whereas acetyl esterase activities were moderate to high in *T. reesei* culture fluid and Lipase PS30, their feruloyl esterase activities were very low. Conversely, Lipase A and Cytolase M102 had over 100-fold greater levels of feruloyl esterase activity relative to acetyl esterase activity. Lipase M had comparable levels of both activities.

In conclusion, the method is feasible for rapid routine assays of samples containing moderate to high levels of feruloyl esterase provided that proteases are absent or at low levels. The problem of protease interference may be avoided by first obtaining independent chromatographic evidence for feruloyl esterase activity and by quantitative protease detection in unknown samples.

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References

[1] B. Bartolomé, C.B. Faulds, M. Tuohy, G.P. Hazlewood, H.J. Gilbert, G. Williamson, Influence of different xylanases on the activity of ferulic esterase on wheat bran, Biotechnol. Appl. Biochem. 22 (1995) 65–73.

[2] C.B. Faulds, B. Bartolomé, G. Williamson, Novel biotransformation of agro-industrial cereal waste by ferulic acid esterases, Ind. Crops Prod. 6 (1997) 367–374.

[3] C. Barbe, D. Dubourdieu, Characterisation and purification of a cinnamate esterase from *Aspergillus niger* industrial pectinase preparation, J. Sci. Food Agric. 78 (1998) 471–478.

[4] P.A. Kroon, C.B. Faulds, C. Brézillon, G. Williamson, Methyl phenylalkanoates as substrates to probe the active sites of esterases, Eur. J. Biochem. 248 (1997) 245–251.

[5] W.S. Borneman, R.D. Hartley, D.S. Himmelsbach, L.G. Ljungdahl, Assay for *trans-p*-coumaroyl esterase using a specific substrate from plant cell walls, Anal. Biochem. 190 (1990) 129–133.

[6] B.L. Garcia, A.S. Ball, J. Rodrigues, M.I. Pérez-Leblic, M.E. Arias, J.L. Copa-Patiño, Induction of ferulic acid esterase and xylanase activities in *Streptomyces avermitilis* UAH30, FEMS Microbiol. Lett. 158 (1998) 95–99.

[7] B.P. Dalrymple, Y. Sadling, D.H. Cybinski, G.-P. Xue, Cloning of a gene encoding cinnamoyl ester hydrolase from the ruminal bacterium *Butyrivibrio fibrisolvens* E14 by a novel method, FEMS Microbiol. Lett. 143 (1996) 115–120.

[8] R.D. Hatfield, R.F. Helm, J. Ralph, Synthesis of methyl 5-*O*-*trans*-feruloyl-α-L-arabinofuranoside and its use as a substrate to assess feruloyl esterase activity, Anal. Biochem. 194 (1991) 25–33.

[9] J. Donaghy, A.M. McKay, Purification and characterization of a feruloyl esterase from the fungus *Penicillium expansum*, J. Appl. Microbiol. 83 (1997) 718–726.

[10] W.S. Borneman, R.D. Hartley, H. Morrison, D.E. Akin, L.G. Ljungdahl, Feruloyl and *p*-coumaroyl esterase from anaerobic fungi in relation to plant cell wall degradation, Appl. Microbiol. Biotechnol. 33 (1990) 345–351.

[11] S. Okamura, M. Watanabe, Measurement of hydroxycinnamic acid ester hydrolase activity by the change of UV absorption, Agric. Biol. Chem. 46 (1982) 297–300.

[12] J.A. McCallum, I.E.P. Taylor, G.H.N. Toers, Spectrophotometric assay and electrophoretic detection of *trans*-feruloyl esterase activity, Anal. Biochem. 196 (1991) 360–366.

[13] M. Mastihubová, V. Mastihuba, L. Kremnický, J.L. Willett, G.L. Côté, Chemoenzymatic preparation of novel substrates for feruloyl esterases, Synlet (2001) 1559–1560.

[14] M. Kordel, B. Hofmann, D. Schomburg, R.D. Schmidt, Extracellular lipase of *Pseudomonas* sp. Strain ATCC-21808, purification, characterization, crystallization, and preliminary X-ray diffraction data, J. Bacteriol. 173 (1991) 4836–4841.

[15] P. Biely, C.R. MacKenzie, H. Schneider, Production of acetyl xylan esterase by *Trichoderma reesei* and *Schizophyllum commune*, Can. J. Microbiol. 34 (1988) 767–772.

[16] P. Biely, G.L. Côté, L. Kremnický, D. Weisleder, R.V. Greene, Substrate specificity of acetylxylan esterase from *Schizophyllum commune*: mode of action on acetylated carbohydrates, Biochim. Biophys. Acta 1298 (1996) 209–222.

[17] M.A. Cotta, R.B. Hespell, Proteolytic activity of the ruminal bacterium *Butyrivibrio fibrisolvens*, Appl. Environ. Microbiol. 52 (1986) 51–58.

[18] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[19] M. Ottesen, I. Svendsen, in: G.E. Perlman, L. Lorand (Eds.), Methods in Enzymology, Academic Press, New York/London, 1970, pp. 199–215.

[20] J.E. Folk, E.W. Schirmer, Chymotrypsin C. I. Isolation of the zymogen and the active enzyme: preliminary structure and specifity studies, J. Biol. Chem. 240 (1965) 181–192.